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6. AUTHOR(S)
Mark Smulson, Ph.D.
Professor of Biochemistry and Molecular Biology
Georgetown University School of Medicine

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7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Georgetown University School of Medicine, Department of Biochemistry and Molecular Biology, 3900 Reservoir Road, Washington, DC, 20057

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9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)
AFOSR
Dr. Walter Kozumbo
801 North Randolph St, Room 732
Arlington, VA, 22203
Teleph: (703)696-7720

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14. ABSTRACT

We observed that exposure of rat lung alveolar type II epithelial cells (RLE-6TN) to JP-8 in vitro induces biochemical and morphological markers of apoptotic cell death such as activation of caspase-3, cleavage of poly(ADP-ribose) polymerase, chromatin condensation, release of cytochrome c from mitochondria, and cleavage of genomic DNA. Generation of reactive oxygen species (ROS) and depletion of intracellular reduced glutathione (GSH) also showed to play important roles in the induction of programmed cell death by JP-8. With the use of macroarray analysis, we further demonstrated that human Jurkat T cells exposed to JP-8 manifested pronounced changes in the expression of genes related to the cellular response to oxidative stress or to apoptosis. In contrast, similar analysis with normal human keratinocytes revealed that exposure to JP-8 induced changes in the expression of genes whose products function in detoxification or regulation of cell growth . These findings might be relevant to the relative resistance of keratinocytes to JP-8 toxicity and support the notion that the induction of cell death by JP-8 may be cell type specific.

15. SUBJECT TERMS
PARP, ADP-ribosylation, JP-8, apoptosis, DNA microarray, comparative genome hybridization (CGH), GSH, reactive oxygen species (ROS)

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1. COVER SHEET

FINAL PERFORMANCE REPORT

THE KEY INVOLVEMENT OF POLY(ADP-RIBOSYL)ATION IN DEFENSE AGAINST TOXIC AGENTS: MOLECULAR BIOLOGY STUDIES

(AFOSR grant FA49620-01-1-0263)

04/01/01 - 03/31/04

PRINCIPAL INVESTIGATOR: MARK E. SMULSON, Ph.D.
PROFESSOR OF BIOCHEMISTRY
DEPARTMENT OF BIOCHEMISTRY AND MOLECULAR BIOLOGY
GEORGETOWN UNIVERSITY SCHOOL OF MEDICINE
3900 RESERVOIR ROAD, N.W.
WASHINGTON, D.C. 20057

FINAL PERFORMANCE REPORT

2. OBJECTIVES:

The prime focus of the past period of this ongoing program has been to test whether components of JP-8 cause permanent genetic instability, as manifested by gains or losses of chromosome regions using CGH, SKY, or changes in gene expressions (DNA micro-array analysis) by JP-8. For all studies in the ***three Aims*** of the grant, we initially intended to utilize tissue culture cells to establish basic experimental parameters. The cells used included RLE-6TN lung epithelial cells, human U-937 monocytic cell line, and human Jurkat T cells. Additionally human keratinocytes, in their differentiated state as a skin layer on nude mice and PARP K/O and control cells and the PARP K/O animal systems were studied. Later studies proposed to utilize either lymphocytes or primary fibroblasts from individuals exposed to JP-8 under occupational conditions. Accordingly, the Objectives and hence Aims of this project are best described by the original Aims, as submitted in 2000 and condensed below.

Aim I: Follow Through on recent, specific data with a variety of cells in culture concerning JP-8 Induction of Cell Death Via Apoptosis and/or Necrosis,

1. Since JP-8 (at $1:10^{-4}$ dilution) causes apoptosis in rat lung, human T, monocytes and other cell cultures in part by caspase-3 activation, we planed to determine the targets of the apoptotic cycle of JP-8 induction "upstream" prior to caspase activation.
2. Caspase-8, -9 were to be tested for their activation by JP-8 and a role for *FAS* receptor was tested.
3. Mitochondria were examined with special emphasis on the pro-apoptotic proteins (i.e. AIF, Bax, Bad), in comparison with the anti-apoptotic factors (i.e. Bcl-2, Bcl-xL).
4. The mode of cell toxicity or death induced by JP-8, in the context of the whole animal (normal and PARP K/O), was examined and the classic apoptotic markers will be examined in lung, brain, and cells of the immune system.
5. The mechanism for the observation that skin keratinocytes and fibroblasts are more insensitive to JP-8 than are RLE-6TN cells, and additionally to explain the apparent JP-8 induced necrosis in the skin cell systems was determined.
6. Whether large-sized DNA strand breaks are induced in keratinocytes exposed to JP-8 and PARP reduces necrosis by a drop in NAD/ATP levels after sensing necrotic DNA strand breaks was also established.
7. We proposed to test whether poly(ADP-ribosyl)ation of p53 is involved in JP-8 induced cell death in keratinocytes and also in lung cell cultures, as controls.

Aim II: Analysis of Chromosomal Abnormalities Induced By JP-8 Exposure Of Cells *In vitro* and in Primary Fibroblasts of Personnel Occupationally Exposed to JP-8: Use of Comparative Genomic Hybridization (CGH) And Spectral Chromosomal Karyotyping (SKY).

JP-8 jet fuel, contains benzene and many other substances, which are suspected of being human carcinogens. Chronic exposure to these agents is associated with blood diseases, such leukemia—which, is well known to be manifested in chromosomal instability and especially translocations. The overall experimental strategy for **Aims II, III** involved the hypothesis that a portion of the toxic effect of JP-8 fuel, or its components, upon non-toxic levels, but long-term exposure causes chromosomal instability as manifested by amplifications, losses or translocations of specific (or random) genes.

1) We proposed to test whether consistent exposure to non-toxic levels of JP-8 manifest cytogenic genomic instability as displayed by unstable ploidy using FACS analysis.

2) Chromosomal genomic gains or losses were to be assessed by CGH.

3) By using PCR analysis, the gene regions of gains and losses would be assigned to established genes, and effects on their mRNA expression examined by RT-PCR and protein expression by Northern analysis.

4) We also proposed to test whether JP-8 causes translocations of regions of chromosomes by both G-banding, as well as "chromosome painting" (i.e. Spectral Karyotyping).

5) Whether well characterized, candidate genes, which are established to be involved in toxicity, are associated with any translocated regions was also proposed to be examined.

Aim III: DNA Microarrays And Toxico-Genomics: "Signature" Alterations In Gene Expression Induced By JP-8.

My laboratory has become involved, on a collaborative basis, with the laboratory of Dr. Peter Schultz (Scripps Institute). Using chips containing over 60,000 mouse genes analysis of mRNA from PARP K/O and control cells has already been performed and compared. The potential applications of DNA array data to toxicological research, "toxo-genomics" and risk assessment of JP-8 will identify gene expression changes and toxic-specific "signatures" for environmental monitoring assays.

1) Using DNA micro-array technology we proposed to test whether JP-8 exposure of cells (or cells from individuals) exposed to JP-8 produce a consistent and definable change in gene expression.

2) The cDNA "ToxChip," (developed at the NIEHS) was be considered to be used for gene expression changes since these chips contain many of the genes noted to become expressed upon toxic insult.

3) The variability of gene expression after JP-8 was assessed in different cells where JP-8 induces toxicity by either apoptosis or necrosis (**Aim I**).

3. STATUS OF EFFORT:

Using differing specialized cells we established how JP-8 causes cytotoxicity via poly (ADP-ribose) polymerase (PARP) and apoptosis. We observed a severe dose and time dependent decrease in cell viability by JP-8 in, rat lung epithelial cell line (RLE-6TN), characterized by caspase 3 activation, PARP cleavage, chromatin condensation and genomic DNA cleavage. Mitochondrial injury followed by the release of molecules including cytochrome c occurs early in apoptosis. Pro-apoptotic, cytochrome c is released from mitochondria after JP-8 exposure, raising the possibility that mitochondrial damage plays a determining role in the induction of apoptosis.

It was observed that monoblastoid (U937) cells undergo apoptosis upon JP-8 exposure as assessed by caspase activation, cleavage of caspase substrates (i.e. PARP, DNA-PK, and Lamin) and cleavage of genomic DNA with the production of HMW fragments and early activation of PARP. Jurkat cells also undergo apoptosis active JP-8 treatment. Significantly, Jurkat cells, transfected with Bcl-xL (an antiapoptotic protein), demonstrate increased survivability when exposed to JP-8.

Since skin is also exposed to JP-8, its cytotoxic effects on human keratinocytes, grown in culture—as well as when grafted onto nude mice—was studied. JP-8 is cytotoxic to human keratinocytes, as evidenced by membrane blebbing, cell rounding and chromatin condensation. Immunoblot analysis further revealed that exposure of keratinocytes to JP-8 markedly downregulates the expression of the anti-apoptotic members of the Bcl-2 family, Bcl-2 and Bcl-xL, and upregulates the expression of pro-apoptotic members of this family, such as Bad and *bax*. Perturbation of the balance between pro- and anti-apoptotic Bcl-2 family members may thus help determine the susceptibility of human keratinocytes to cell death induced by JP-8 fuel.

Furthermore, immortalized fibroblasts derived from $PARP^{-/-}$ mice exhibited an attenuated JP-8 response compared with $PARP^{-/-}$ cells, stably transfected with PARP cDNA, indicating that the JP-8 mediated alterations in the levels of these Bcl-2 family proteins are also partially poly(ADP-ribose) polymerase (PARP)-dependent. Finally, when human keratinocytes were grafted to form a human epidermis on nude mice, treatment of these grafts with JP-8 revealed cytoxicity and altered histology *in vivo*.

4. ACCOMPLISHMENTS/NEW FINDINGS:

A. PROGRESS ON GENE EXPRESSION BY MACRO AND AFFYMETRIX MICRO ARRAY ANALYSIS: RELEVANCE TO JP-8 TOXICITY.

Espinoza, L.A., **Smulson, M.E.** Macroarray Analysis of the Effects of JP-8 Jet Fuel on Gene Expression in Jurkat Cells. *Toxicology.* 189(3):181-90 2003.

Exacting analysis has been asserted to identify specific genes that correlate both with mRNA expression as well as, in collaboration with Frank Witzmann, proteomics, as good candidates to focus on in therapeutic strategies towards JP-8 toxicity. During the past period before we were positioned to be able to utilize Affymetrix chip analysis due to priority availability of equipment. Additionally, a study was made utilizing macro arrays produced by Clonetech. Accordingly, the effects of exposure of human T-cells (Jurkat) to JP-8 at a 1 $x10^{-4}$ dilution for 4 h on gene expression were examined using this technology. We had previously shown in these cells that under the above conditions, JP-8 causes significant apoptosis markers to appear by 4 h and death by 24 h. Of the 439 apoptosis-stress response-related genes examined with the Clontech membranes in hybridization, the expression of 16 genes was shown to be up-regulated and that of 10 genes was down-regulated by a factor of > 2.

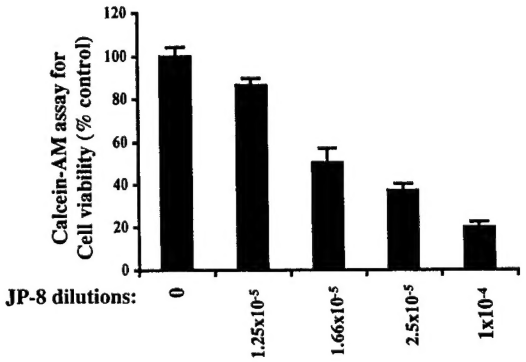
Smulson, M.E., Espinoza, L., Boulares, H.A., Haddad, B.R., Witten[1], M., and Hyde[1], J. Department of Biochemistry and Molecular Biology, Department of Oncology, Georgetown University School of Medicine, Washington, DC [1]Department of Pediatrics and Cell Biology and Anatomy, The University of Arizona, Tucson, Arizona. Abstract **AFOSRJP-8 Jet Fuel Toxicology Workshop 14-16 May 2003.**

With respect to the second focus on JP-8, our initial results using Affymetrix oligomicroarray technology to determine changes in gene expression in rat lung tissue after JP8 inhalation treatment were performed. In collaboration with Dr. Mark Witten, rats were exposed to JP8 for 7 days at 352 mg/m^3, by inhalation at one hour per/ day. Lungs and various tissues were sent to our laboratory; each time point was analyzed in quadruplicate. mRNA was prepared and processed for hybridizaton to microarray chips. Of the 24,000 genes and 7,000 EST monitored on the Affymetrix rat chips, 76 genes were significantly altered (PE<0.05) with a fold greater than 1.5 by JP-8. For example, the gamma-synuclein gene, which is associated with various cancers was observed to be five fold up-regulated. Significant increases were detected in the expression of genes involved in stress response and GST-transferases. On the other hand, transcripts related to cell signaling and transcriptional factors were down-regulated. Currently, we are analyzing spleen RNA from the same experiment. Additionally, rat exposure experiments have been performed, but not yet analyzed utilizing 100 mg/m^3 JP-8 exposure to coincide with Frank Witzmann's proteomic data.

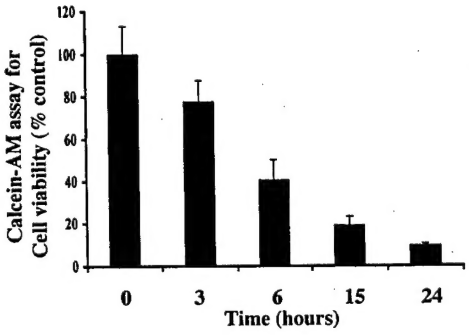
B. ONGOING STUDIES ON THE MOLECULAR MECHANISMS FOR JP-8 INDUCED CELL KILLING IN RAT LUNG EPITHELIAL CELLS, AND VARIOUS HUMAN CELLS.

Stoica, B.A., Boulares, A.H., Rosenthal, D.S., Iyer, S., Hamilton, I.D.G., **Smulson, M.E..** Mechanisms of JP-8 Jet Fuel Toxicity: I. Induction of Apoptosis in Rat Lung Epithelial Cells. *Tox. Appl. Pharmacol.* 171: 94-106 (2001)

JP-8 jet fuel induces cell death in rat lung laveolar type II epithelial cells (RLE-6TN) in a dose and time dependent manner

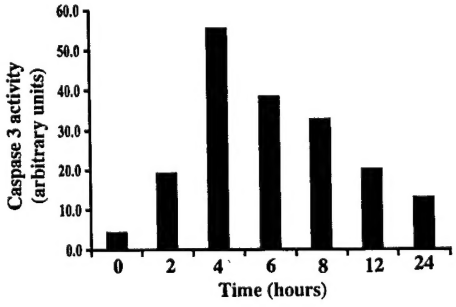


(Fig 1A)

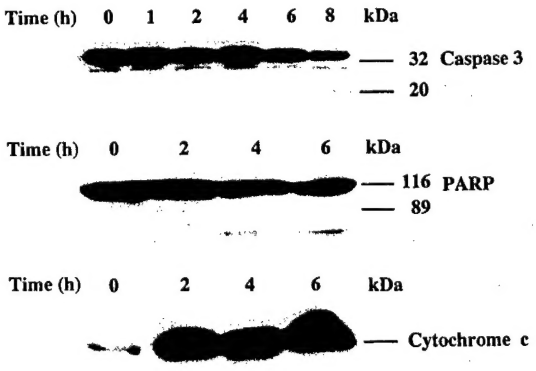


(Fig 1B)

Exposure of RLE-6TN to JP-8 jet fuel induces caspase-3 activation, PARP cleavage, and cytochrome *c* release into the cytoplasm



(Fig 2)



(Fig 3)

Inhalation is one of the primary routes of JP-8 exposure; therefore we decided to establish an *in vitro* model that will allow the dissection of the mechanisms underlying the JP-8 jet fuel toxicity in a cell line representative of the respiratory system. RLE-6TN is a cell line that originated from rat alveolar epithelial cells and preserves many of the phenotypic characteristics of the original alveolar cells. It was observed that JP-8 induces a dose-dependent decrease in cell

viability in the RLE-6TN cells after 24h of exposure at the indicated dilutions of JP-8. A dilution of $1x10^{-4}$ JP-8 is able to kill virtually all the cells (***Fig 1 A***). During a time-course exposure to a $1x10^{-4}$ dilution of JP-8 jet fuel, we noted a rapid decrease in cell viability with a little more than 50% of the cells being dead after six hours of exposure to JP-8 (***Fig 1B***).

To gain further insight in the type of cell death induced in RLE-6TN cells upon exposure to $1x10^{-4}$ dilution of JP-8 jet fuel, we utilized phase contrast microscopy during a time course of treatment. The cells became rounded between 2 h and 4 h; this was followed by membrane blebbing, fragmentation and detachment from the substrate after 6 h. All these indicating that apoptosis was one form of death due to components of JP-8 at very low dilutions. Caspase-3 activity was induced at 2 h and peaks at about 4 h of exposure after which a gradual decrease of caspase-3, a key marker for apoptosis was observed (***Fig 3C***). Caspase-3 activation from its proenzyme of 32kDa (p32) occurs with a cleavage into a 20 kDa (p20) and 12kDa (p12) subunit. The data in (***Fig 3A***) employed specific antibodies for these cleavage products. The presence of its p20 subunit was detected as early as 4h after JP-8 treatment. **Mitochondrial injury is an important inducer of apoptosis**, and considerable experiments have been performed over the last three years. During apoptosis mitochondria release cytochrome c from the intermembrane space into the cytosol where it can activate caspase-9, via Apaf I and ATP/dATP. According we examined the release of cytochrome c into the cytosol after exposure of the lung cells to JP-8; cytochrome c levels in cytosol were observed as early as 2h after treatment with JP-8 (***Fig 3C***). This finding indicates that mitochondrial damage is one of the earliest manifestations of JP-8 toxicity and occurs early.

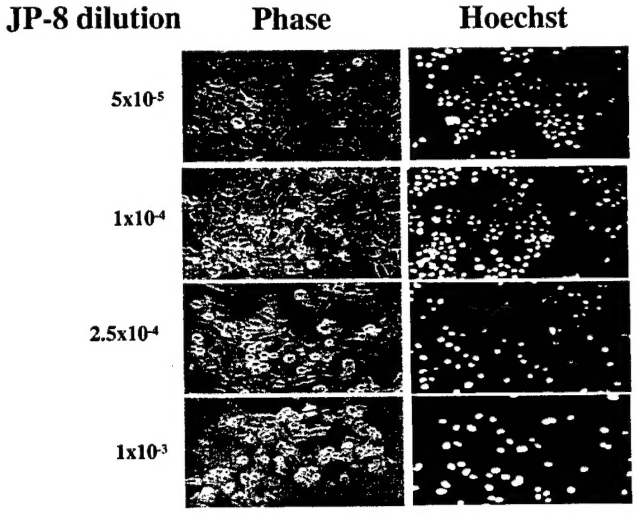
The human monoblastoid cell line (U-937) responds to JP-8 exposure with caspase-3 activation, cleavage of caspase substrates like PARP, DNA-PK, and lamin B1 and degradation genomic DNA with the production of HMW fragments. We showed in this manuscript that a caspase-3 like activity is detectible using dilution of $1x10^{-4}$ as early as 1h. The cleavage of PARP was also noted after 1h of treatment as well as lamin B1, which is a substrate for caspase-6 during apoptosis. The cleavage of lamin B1 from its normal size of 64kDa into 46kDa occurs as early as 2h. Additionally, DNA-PK, which is an enzyme involving DNA repair and recombination is another substrate for caspase-3 was cleaved very early.

The same types of relationships noted above also were induced in Jurkat-T cells leukemia cells treated with JP-8. It was interesting that Jurkat stability transfected with the plasmid encoding in the anti-apoptotic protein Bcl-xL or when pre-treated with the DBAC FMK-caspase inhibitor demonstrated **increased survivability** when exposed to JP-8.

Rosenthal, D.S., Simbulan-Rosenthal, C.M.G., Liu, W.F., Stoica, B.A., and Smulson, M.E.. Mechanisms of JP-8 Jet Fuel Cell Toxicity: II. Induction of Necrosis in Skin Fibroblasts and Keratinocytes and Modulation of Levels of Bc1-2 Family Members. *Tox. Appl. Pharmacol.* 171: 107-116 (2001).

In this study we observed a different mechanism of cytotoxicity by JP-8 on human keratinocytes grown in culture, as well as when grafted onto nude mice. At the lower level of JP-8 ($1x10^{-4}$ dilution), which was sufficient to induce apoptosis in the cells discussed above no apoptosis, was observed in either immortalized (Imm HEK) or **primary** (1^0 HEK) cells.

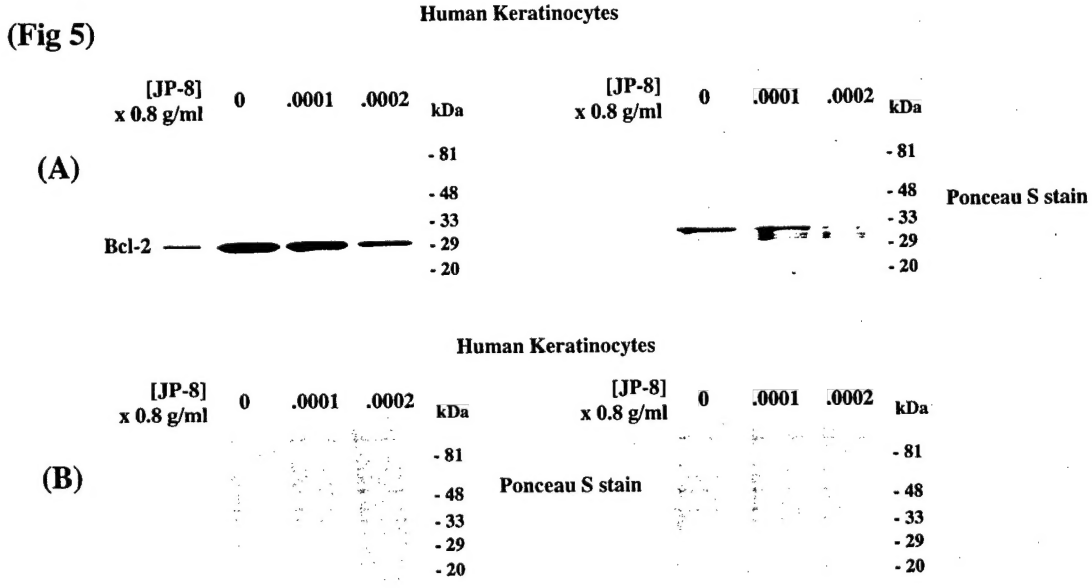
JP-8 induces necrotic cell death in human epidermal keratinocytes



(Fig 4)

JP-8 reduces the levels of pro-survival members of the Bcl-2 family (Bcl-2 and Bcl-XL) in human epidermal keratinocytes

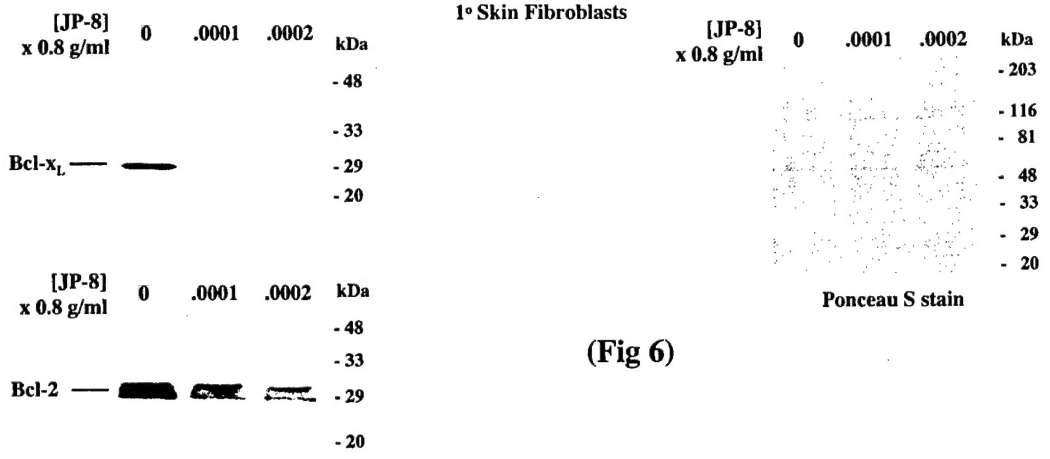
(Fig 5)



At higher doses (>2.5x10^{-4} dilution), JP-8 is cytotoxic to both primary and immortalized human keratinocytes, as evidenced by the metabolism of calcein. Rather, morphological changes characteristics of necrosis such as cell rounding and cell detachment was observed (***Fig 4***). FACS analysis indicated that the majority of toxicity resulted from necrosis rather than apoptosis

at these levels. In addition, we did not detect evidence of activation of caspases-3, -7, and -8 by enzyme activity or immunoblot analysis.

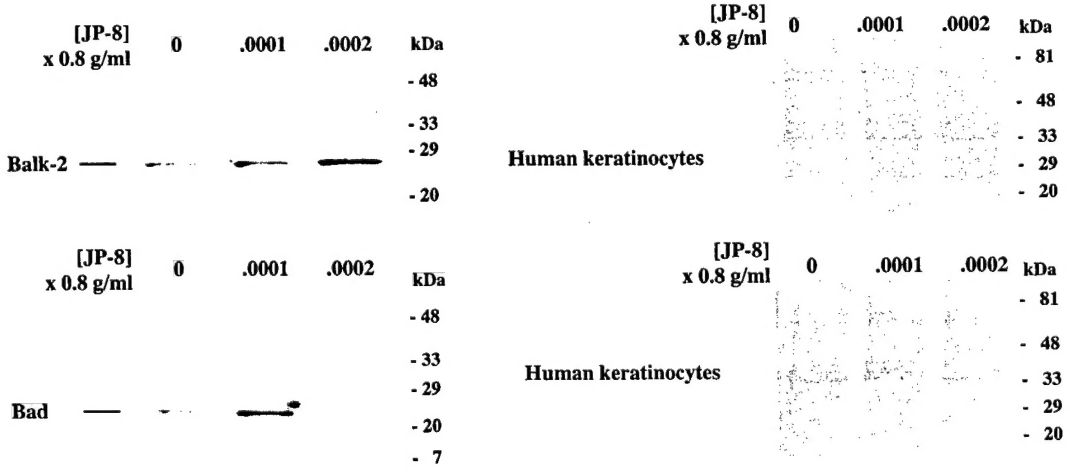
Levels of of the pro-survival proteins Bcl-2 and Bcl-XL are also reduced in primary skin fibroblasts exposed to JP-8



(Fig 6)

Immunoblot analysis revealed that exposure of keratinocytes to the toxic higher levels of JP-8 markedly down-regulates the expression of the pro-survival members of the Bcl-2 family, Bcl-2 and Bcl-xL, (***Fig 5 and 6***) and up-regulates the expression of anti-survival members of this family, including Bad and Bak (***Fig 7***). Bcl-2 and Bcl-xL have been shown to preserve mitochondrial membrane integrity and suppress cell death. In contrast, Bak and Bad, both promote cell death by alterations of the mitochondrial membrane potential, in part by heterodimerization with an inactivation Bcl-2 and Bcl-xL, and either inducing necrosis, or activating a downstream caspase program.

Levels of of the pro-death members of the Bcl-2 family (Bad and Bak) are induced in human keratinocytes exposed to JP-8



(Fig 7)

Topical JP-8 exposure results in gross histological abnormalities of human keratinocytes grafted onto nude mice

Control **JP-8 48-h**

(Fig 8)

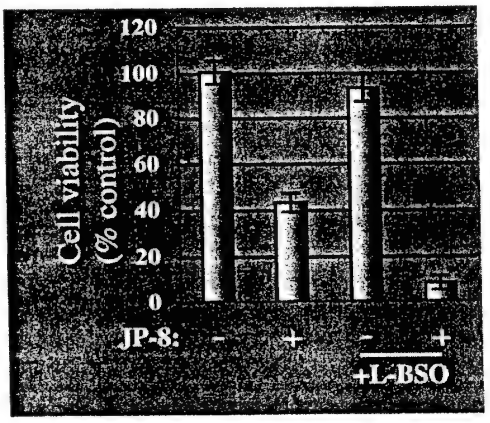
Utilizing the grafting system as previously described, we grafted Nco cells on nude mice to form a histologically and immunocytochemically normal human epidermis. Fifty ml of JP-8 was then applied topically to the graft site. ***Fig 8*** shows the result of an experiment in which control tissue, or tissue treated with topical JP-8, was isolated and subjected to histological analysis. In contrast to the normal morphology of control grafts, JP-8 treated skin showed gross histological abnormalities characterized by severe necrosis of all epidermal layers.

Boulares AH, Contreras FJ, Espinoza LA, **Smulson ME**. Roles of oxidative stress and glutathione depletion in JP-8 jet fuel-induced apoptosis in rat lung epithelial cells. Toxicol Appl Pharmacol. Apr 15;180(2):92-9 (2002).

Utilizing the rat lung epithelial cell line the mechanism of JP-8 toxicity was further investigated in attempt to identify potential therapeutic interventions. Given that oxidative stress changes in concentrations endogenous antioxidants, such as glutathione (GSH), have been associated with cellular damage by numerous toxicants. The possibility that JP-8 induces cellular oxidative stress was examined. We therefore investigated whether the concentration of GSH is relevant to JP-8 toxicity in these lung cells. GHS was depleted from RLE-6TN cells by incubation for 20 h with 200 mM L-BSO, which selectively inhibits g-glutamylcysteine synthetase, the rate-limiting enzyme in GSH biosynthesis. The cells were then treated with JP-8 for 6 h, after which cell viability was assessed. L-BSO markedly potentiated JP-8-induced cell death (***Fig 9***); whereas JP-8 alone induced and ~60% decrease in cell viability.

Glutathione depletion by L-BSO Increases JP-8-induced Cell Death

(Fig 9)



Thiol antioxidants are protective against JP-8-Induced Cell Death

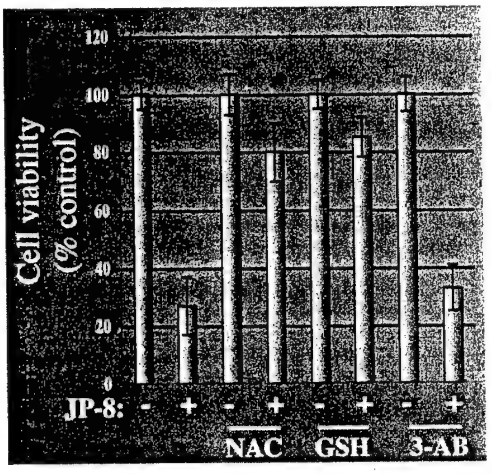
Cells pretreated ± 5 mM NAC, 1 mM GSH, or 5 mM 3-AB for 1 h

↓

Exposure to JP-8 (10^{-4}) for 12 h

↓

Calcein staining (Cell viability)



(Fig 10)

The cells were then treated with JP-8 and L-BSO resulted in >90% cell death (***Fig 9***). It is noteworthy that L-BSO alone had no significant effect on cell viability. Both NAC and GSH were shown to significantly protect the cells from JP-8 loss of viability (***Fig 10***). We examined the direct effects of JP-8 exposure on intracellular GSH levels after 1 h exposure to the fuel. JP-8 significantly reduces the level of GSH (~40%) in RLE-6TN cells, after 1 h of exposure, a time during which no cell death occurs (***Fig 11***).

Protective Effect of Thiol antioxidants against JP-8-Induced Cell Death is Associated with Maintenance of intracellular Concentrations of GSH

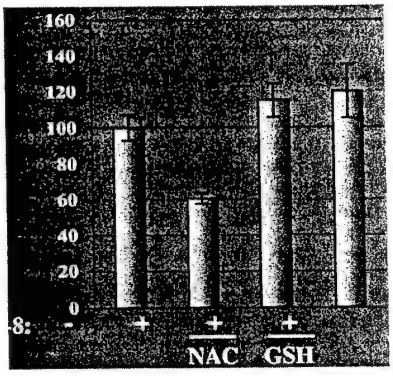
Cells pretreated ± 5 mM NAC, 1 mM GSH, or 5 mM 3-AB for 1 h

↓

Exposure to JP-8 (10^{-4}) for 1h

↓

Assessment of intracellular GSH levels



(Fig 11)

Our results suggest that JP-8 exposure induces oxidative stress in RLE-6TN cells. To test this hypothesis, we utilized H_2O_2 under a specific incubation protocol. Results indicated that while JP-8 alone induced only an ~10% loss of cell viability a combination of JP-8 and H_2O_2 resulted in >60% cell death, whereas treatment with H_2O_2 alone was not significant. We used H_2DCF, an intracellular probe for ROS. Exposure of the lung cells to JP-8 for 2 h triggered oxidation of H_2DCF to the fluorescent DCF moiety, as revealed flourometrically or also by florescent microscopy indicating that JP-8 induces the generation of ROS (***Fig 12***). Additionally, mitochondrial membrane potential was reduced by JP-8 (***Fig 13***) and this was also protected by thiol antioxidants (***Fig 14***).

JP-8 induces the generation of of ROS

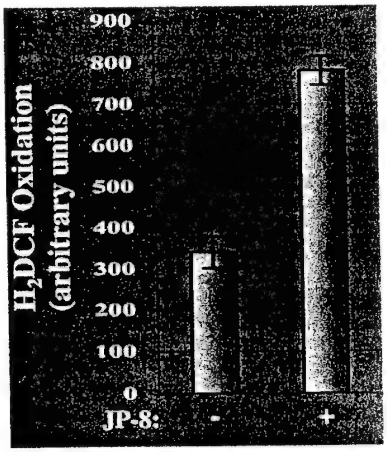
Cells loaded with 50 mM H_2DCF
(fluorescent only when oxidized by ROS)
for 30 min

↓

Assessment of DCF (fluorescent)

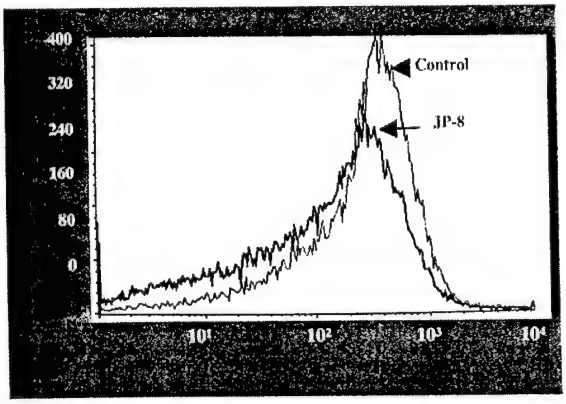
↓

Exposure to JP-8 (10^{-4}) for 2 h

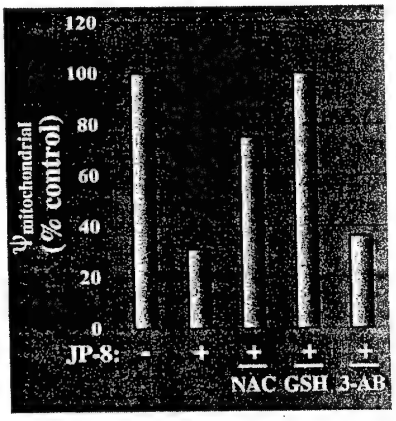


(Fig 12)

Thiol antioxidants but not 3-AB are protective against JP-8-Induced Loss of Mitochondrial Membrane Potential



(Fig 13)



(Fig 14)

Boulares, A.H., Zoltoski, A.J., Sherif, Z., Jolly, P., Massaro, D., and **Smulson, M.E.** Gene Knockout or Pharmacological Inhibition of Poly(ADP-ribose) Polymerase-1 Prevents Lung Inflammation in a Murine Model of Asthma *Am. J. Respir. Cell Mol. Biol.*: 28(3):322-9, 2003

Airway inflammation is a central feature of **JP-8** as well as asthma and chronic obstructive pulmonary disease. Reactive oxygen species (ROS) contribute to inflammation by damaging DNA, which, in turn, results in the activation of PARP-1 and **depletion** of its substrate, NAD. Here, we show that prevention of **PARP-1 activation by DNA strand breaks protects against both ROS-induced airway epithelial cell injury** in vitro and airway inflammation in vivo. H_2O_2 induced the generation of ROS, PARP-1 activation and concomitant NAD/ATP depletion, and release of LDH in A549 human airway epithelial cells. These effects were blocked by the PARP-1 inhibitor 3-AB. Furthermore, 3-AB inhibited both activation of the proinflammatory transcription factor nuclear factor NF-kappaB and expression of the interleukin-8 gene due to DNA breaks induced by H_2O_2 in these cells. In a murine model of allergen-induced asthma. 3-AB prevented airway inflammation possibly caused by JP-8 elicited by ovalbumin. Moreover, **PARP-1 knockout mice were resistant** to such ovalbumin-induced inflammation. These protective effects were associated with an inhibition of expression of the inducible nitric oxide synthase. Thus no DNA breaks, PARP activation and drop in NAD/ATP. These results implicate PARP-1 activation in airway inflammation, and suggest this enzyme as a potential target for the development of new therapeutic strategies in the treatment of asthma as well as other respiratory disorders such as **chronic obstructive pulmonary disease, or those caused by JP-8.**

C. COMPLEMENTARY STUDIES ON THE PARTICIPATION OF PARP1 AND P(ADP-R)^N WITH EARLY STAGES OF APOPTOSIS: STUDIES OF DNA FRAGMENTATION FACTOR (DFF), P(ADP-R)^N, AND 50-100 KB DNA BREAKS.

In the manuscript outlined above by Stoica *et. al.* we showed that JP-8 induces genomic DNA degradation and PARP1 activation in U937 cells. Thus, a brief summary of several papers performed during the last period on this peripheral but relevant topic (*and is acknowledged for partial AFOSR support, as are all the manuscripts in* ***Accomplishment/New Findings***).

Boulares, A.H., Zoltoski, A. J., Yakovlev, A.G., Xu, M., **Smulson, M.E.** Roles of DNA fragmentation factor and poly(ADP-ribose) polymerase in an amplification phase of TNF-induced apoptosis. *J. Biol. Chem.* 276(41): 38185-92 (2001)

During early apoptosis, DNA fragmentation factor (DFF) cleaves DNA into 50- to 300-kb fragments and subsequently into internucleosomal fragments (i.e. coincident with the brief, but critical PARP1 "burst"). DFF factor comprises DFF45 and DFF40 subunits; DFF45 acts as a protective chaperone, as well as an inhibitor of the catalytic subunit, whose cleavage by caspase-3 results in DFF activation. Disruption of the DFF45 gene blocks DNA fragmentation since DFF-40 loses an inhibitor but becomes unstable, and resistant to apoptosis. We investigated the relationships between p(ADP-R)^n and DFF-mediated early DNA fragmentation during apoptosis in primary fibroblasts from DFF45$^{(-/-)}$ and control (DFF45$^{(+/+)}$) mice. TNF induces rapid cleavage of DNA into ~50-kb fragments in DFF45$^{(+/+)}$ fibroblasts but not in DFF45$^{(-/-)}$ cells.

We found that the TNF–induced early activation of PARP1, which requires PARP1 binding to the 50kb DNA strand breaks, and the consequent *depletion of the PARP1 substrate NAD were markedly delayed in DFF45$^{(-/-)}$ cells*, suggesting a role for DFF in this early PARP1 activation during apoptosis. The somewhat later activation of caspase-3, the loss of mitochondrial membrane potential, and the release of cytochrome *c* were similarly delayed in DFF45$^{(-/-)}$ fibroblasts. We noted that inhibition of PARP1 by 3-AB partially protected DFF45$^{(+/+)}$ cells against TNF–induced death as well as inhibited in the associated release of **cytochrome c** and activation of caspase-3. These results suggested to us that the generation of 50-kb DNA fragments by DFF, together with the activation of PARP1, **mitochondrial dysfunction**, and caspase-3 activation, contributes to an **p(ADP-R)^n amplification loop** early in the death process. This was further verified in the subsequent paper.

Boulares,A.H. ,Zoltoski, A.J.,Zaki, A. S, Yakovlev, A., and **Smulson, M. E.** Roles of DNA fragmentation factor and poly(ADP-ribose) polymerase 1 in sensitization of fibroblasts to tumor necrosis factor-induced apoptosis *Biochem. Biophys. Res. Com.*, 290:796-801(2002)

The next series of experiments were designed to verify the hypothesis of the existence of an amplification loop during apoptosis involving PARP1-activation, **mitochondria dysfunction**, and activation of caspase-3. Accordingly, we examined the effects of restoring DFF expression in DFF 45 $^{(-/-)}$ fibroblasts. We showed that co-transfection of mouse DFF45 $^{(-/-)}$ fibroblasts with plasmids encoding human DFF40 and DFF45 reversed the *apoptosis resistance* normally observed in these cells. DFF45$^{(-/-)}$ cells gained the ability to cleave DNA into 50kb fragment in response to TNF; these large breaks resulted in: 1) a marked activation of PARP-1 and a concomitant depletion of NAD, 2) an increase in cytochrome c release into the cytosol and 3) caspase-3 activation.

Smulson, M.E., Espinoza, L., Boulares, H.A., Haddad, B.R., Witten[1], M., and Hyde[1], J. Department of Biochemistry and Molecular Biology, Department of Oncology, Georgetown University School of Medicine, Washington, DC [1]Department of Pediatrics and Cell Biology and Anatomy, The University of Arizona, Tucson, Arizona. Abstract AFOSRJP-8 Jet Fuel Toxicology Workshop 14-16 May 2003.

Given the close association of oxidative stress with intracellular calcium we surmise that JP-8 uninduced intranuclesomal DNA fragmentation may be calcium dependant. Presented at the Annual Air Force Workshop we demonstrated that JP-8 induced DNA fragmentation in U937 cells is closely dependant on intracellular, but not extra-cellular calcium. DNA fragmentation in U937 cells was observed only in concentrations of JP-8 that induce apoptosis but not at those inducing necrosis. This differential induction of intra-nuclesomal DNA fragmentation was associated with a level ofJP-8 induced oxidative stress suggesting a threshold concentration, of which JP-8 treatments which is from apoptosis to necrosis. In fact, pretreatment of cells with a low concentration of H_2O_2 completely inhibited JP-8 induced DNA fragmentation triggering apoptosis, a specific mediator of JP-8 induced internucleosomal DNA fragmentation, which was also sensitive to level JP-8 induced oxidative stress. These results were confirmed by the use of cells sufficient in DNAS1L3 that were transfected with plasmid encoding the human DNAS1L3 gene. Together these results strongly indicate that the differential induction of apoptosis and the resulting DNA fragmentation is closely linked to the level oxidative stress induced by JP-8 exposure.

D. INFLUENCE OF P(ADP-R)^N AND/OR PARP1 ON INTERACTION WITH DNA OF P53 AND E2F1. DNA-PHOBICITY OF P(ADP-R)^N-P53 AND E2F1 AND INTERACTION WITH THEIR PROMOTERS DURING APOPTOSIS.

Simbulan-Rosenthal, C., Rosenthal, D.S., **Smulson, M.E.**, Poly (ADP-ribosyl)ation of p53 During Apoptosis in Human Osteosarcoma Cells. Cancer Res 59:2190-2194, (1999)

In this earlier study on p53 we quantitatively measured the extent and PAR chain length of p(ADP-R)^n-p53 using the slow (10-days) spontaneous apoptosis of osteoscarcoma cells: This data represented the first proof that p53 per se is p(ADP-R)^n *in vivo* and that p53 is significantly expressed during 2-9 days of the spontaneous apoptosis. However, p53 becomes heavily p(ADP-R)^n only during the early stages of apoptosis, p53 loses its chains of PAR during days of apoptosis days 5-9. We noted that at day 3 when p53 is heavily p(ADP-R)^n it cannot bind to consensus promoter sequences using an *in vitro* gel-shift assay. However, p53 induced later in apoptosis when p53 is not p(ADP-R)^n, there is induction of the pro-apoptotic proteins *Bax* and *Fas*, which occurs at exactly the same time that is p(ADP-R)^n cleaved from p53. **This aspect of p53 and PARP will be related to DNA strand breaks and mechanisms of induction of apoptosis induced by JP-8 in a renewed application to be submitted.**

Simbulan-Rosenthal C.M.,Rosenthal D.S., Luo, R., Samara, R., Jung, M., Dritschilo, A., Spoonde, A., Smulson, M.E. Poly(ADP-ribosyl)ation of p53 In Vitro and In Vivo Modulates Binding to its DNA Consensus Sequence. Neoplasia. 3:235-244 (2001)

Purified wild-type recombinant PARP1 were shown to catalyze the p(ADP-R)^n of full-length p53 *in vitro (This study is the in vitro support for the Cancer Res. studies above).* In gel-super shift assays, p(ADP-R)^n **suppresses** p53 binding to its DNA consensus sequence. However, when p53 remain unmodified in the presence of an inactive mutant PARP-1 it retains sequence specific DNA binding activity. In extracts prepared from osteosarcoma cells during early apoptosis. Using super shifts and isolated extracts from the above *in vivo* experiments we observed that p53 interaction with its DNA consensus sequence was only operative when the polymer had been cleaved from the tumor suppressor.

Mandir, A.S., Simbulan-Rosenthal, C.M., Poitras, M.F., Lumpkin, J.R., Dawson, V.L., **Smulson, M.E.**, and Dawson, T.M. "A Novel In Vivo Post-Translational Modification of p53 by PARP1 in MPTP-Induced Parkinsonism" *J. Neurochem.* Oct; 83(1): 186-92 (2002).

In this on going collaboration with the Department of Neuroscience at John's Hopkins University Medical School, we have now **further characterized new roles for p(ADP-R)^n of p53** on MPTP-induced Parkinsonian disease in PARP-1$^{(+/+)}$ and PARP-1$^{(-/-)}$ mice. In an earlier paper we showed that mice lacking the gene for PARP-1 were dramatically spared from MPTP neurotoxicity by preventing neuronal depletion of NAD and ATP. MPTP potentially activates PARP-1 (via nitric oxide synthesis and resultant DNA breaks) in vulnerable dopamine containing neurons. Our laboratory showed in an earlier study that MPTP elicit a novel pattern of p(ADP-R)^n of nuclear proteins that completely depends on neuronally derived nitric oxide via nNOS. We observed that a large modification of protein of approximately 50 to 60 kb occurred in these experiments, and this led to the more recent collaboration above, which further contributes to a

progress on understanding the complex roles of p(ADP-R)^n in different systems which might induce JP-8 toxicity.

We suspected that the heavily modified nuclear protein in the initial study might be p53. Our laboratory showed by immunoprecipitation that, in fact, p53 is extensively p(ADP-R)^n by PARP-1 following MPTP induction of Parkinson's Disease. We believe that this high level of p(ADP-R)^n products a large negative charge on p53 which alters it's transactivation of downstream genes during the onset of this disease (a major hypothesis of this **AFOSR Renewal with respect to JP-8 Aim II**). Thus, the influences of PARP-1 on p53 noted here may underlie a mechanism of MPTP-induced Parkinsonism and other models of neuronal death.

Simbulan-Rosenthal, C.M., Rosenthal, D.S., Luo, R., Samara, R., Espinoza, L.A., Hassa, P.O., Hottiger, M.O., and **Smulson, M. E.,** PARP-1 Binds E2F-1 Independently of its DNA Binding and Catalytic Domains, and Acts as a Novel Co Activator of E2F-1-Mediated Transcription During Reentry of Quiescent Cells into S-phase, *Oncogene* 22:8460-71, 2003.

In earlier work using PARP KO and wild-type cells we were able to show that PARP-1 up-regulates E2F-1 promoter during S-phase reentry by DNase. In contrast to our studies described above that p53, an acceptor for the p(ADP-R)^n reaction, we found that E2F-1 was not p(ADP-R)^n by wild-type recombinant PARP-1, in vitro. Co-immunoprecipitation experiments with purified PARP-1 and E2F-1, however, revealed that **PARP-1 binds to E2F-1** *in vitro* and **also** *in vivo*. In wild-type fibroblasts 7 h after reentry into S phase, coincident with the increase in E2F-1 promoter activity there is expression of E2F-1-responsive S-phase genes such as cyclin A and DNA pol α. Mapping of the interaction domains of PARP we observed a **PARP-1 mutant** lacking the auto-modification domain does not bind thus, *indicating that the protein interaction site is in this central domain*. Finally, gel supershift analysis with end-blocked E2F-1 promoter sequence probes verified that *binding of PARP-1 to E2F-1 markedly enhances binding to the E2F-1 promoter, suggesting that PARP-1 may act as a positive cofactor of E2F-1 mediated transcription.*

5. PERSONNEL SUPPORTED:

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6. PUBLICATIONS:

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Some publications included contributed scientifically to the project and were accordingly acknowledged as being partially supported by F49620-01-1-0263 in each paper

Espinoza, L.A., Li, P., Lee, R.Y., Wang, Y., Boulares, A.H., Clarke, R., **Smulson, M.E.** Evaluation of gene expression profile of keratinocytes in response to JP-8 jet fuel. *Toxicol. Appl. Pharmacol.* 200:93-102, 2004.

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Selected Invited Chapters

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